DRAFT (Version 2.0) December 20, 1999

Streptococcus pneumoniae opsonophagocytosis using differentiated HL-60 cells

(Promyelocytic Leukemia Cell Line)

Laboratory Protocol prepared by:

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APPENDICES

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Figures and Tables are not available in the electronic version, if interested a hard copy it can be mailed to you upon request to:

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I. OVERVIEW

A standarized opsonophagocytic assay is described that reproducibly estimates the "functional" phagocytic activity of human serum for *S. pneumoniae* (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F have been tested). This assay measures the complement dependent opsonic activity of serum using culturable phagocytic cells. The HL-60 promyelocytic leukemia cell line is used as effector cells instead of peripheral blood leukocytes (PBLs). Phagocytosis is determined by viable counts of *S. pneumoniae*. Opsonophagocytosis measures functional antibody, as opposed to ELISA, which measures total binding antibody (functional and non-functional). This assay can be used for evaluating the human response to current and developing pneumococcal vaccines.

II. BACKGROUND INFORMATION

Host protection against pneumococcal disease is mainly mediated by phagocytosis (14, 16). Opsonophagocytosis is a mechanism by which the host protects against infection, with the participation of serum opsonins (antibodies and complement). The presence of functional antibodies leads to an effective opsonization and recovery from infection (see Appendix G, pg. 29).

Opsonophagocytic assays for *S. pneumoniae* are traditionally performed using PBLs as effector cells. A variety of techniques have been used to determine opsonophagocytic activity (radioisotopes, chemiluminesce, flow cytometry and viability assays), however a standardized assay is not available. With the use of culturable phagocytes of the granulocytic lineage, it is possible to reproducibly estimate the phagocytic titer of sera from vaccinated and unvaccinated individuals that have received either the polysaccharide vaccine or an investigational polysaccharide protein-conjugate vaccine (7, 11).

The HL-60 cell line originated from an individual with promyelocytic leukemia and was established as a continuous cell line by Collins *et al.* in 1977 (3). The main characteristic of this cell line is that it is composed of undifferentiated hemapoietic cells consisting of 85 % blasts and promyelocytes, 6% myelocytes, and 9% differentiated cells. These cells are capable of undergoing differentiation into various cell lineages. The undifferentiated cells undergo differentiation upon chemical induction (1, 2). The granulocytic differentiation induced by N,N-dimethylformamide yields polymorphonuclear-like cells (44% myelocytes and metamyelocytes, and 53% bands and PMNs). The granulocytic differentiation induced by polar compounds was described by Collins in 1978 (4). The use of differentiated HL-60 cells in the opsonophagocytosis assay decreases the variability observed among donors and likely will decrease within and between laboratory assay variability for vaccine evaluation.

III. SUMMARIZED PROTOCOL

1. Add 10 µl of Opsono buffer to each well of a 96-well plate except for row A.

2. Add 20 µl of diluted or undiluted test serum to the wells of row A. Serum is heat inactivated at 56^{o}C for 30 min prior to testing. A9 through A12 are reserved for QC sera (serum with a known titer and Sandoglobulin).

3. Serially dilute (2-fold) test serum (rows A through H), except wells H9 through H12, which are reserved for the C' control.

4. Add 20 µl of bacterial suspension to each well (1,000 cfu/well). Bacteria come from a frozen stock (10^8 cfu/ml) that was serially diluted in Opsono buffer to obtain 1,000 cfu in 20 µl. This suspension is kept on ice until ready to use.

5. Incubate at 37^{o}C for 15 min, 5% CO_2.

6. Add 10 µl of complement source (Baby Rabbit Serum). Serum is kept frozen at $\leq$-70^{o}C in 1 ml aliquots until ready to use. A small amount (100 µl) is heat inactivated at 56^{o}C for 30 min for appropiate controls, when necessary.

7. Add 40 µl of differentiated HL-60 cells to each well (4×10^5 cells/well). Cells are pre-washed once in Hanks buffer and resuspended in Opsono buffer at room temp.

8. Incubate at 37^{o}C for 45 min with horizontal shaking (220 rpm) in room air.

9. Cool on ice for 1 min to stop the reaction and bacterial growth, if more than two plates are being run.

10. Plate 5 µl from each well onto THYE plates. Spot plating one column at a time.

11. Allow spots to air dry (~5 min) and incubate overnight at 37^{o}C, 5% CO_2.

12. Count colonies after ~18 h to avoid overgrowth. Compare count to the C' control count which corresponds to the number of bacteria present in each well after a 45 min incubation period in the presence of the complement source.

13. Complement growth control wells (H9 through H12) contain 20 µl Bacteria, 10 µl complement, 10

µl HL-60 cells and 10 µl Opsono buffer.

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IV. OPSONOPHAGOCYTIC ASSAY

A. Differentiated HL-60 Cells

1. Harvesting

a. Perform a viable cell count on each of the 200 ml cultures (T-150 flasks). See Appendix , pg. 27.

b. Calculate the number of cells required for running one plate. See Note 1, pg. 30.

c. Centrifuge the total volume required for 10 min at 160xg at room temperature.

d. Discard the medium by inverting tube with a single motion and drain excess medium by touching the edge onto a clean paper towel (lint-free) without returning

tube to the upright position.

e. Once drained, each pellet is resuspended in 5 ml of Hanks (Reagent M).

1) Use Hanks (Reagent M) at room temperature.

2) Use plastic pipets only (cells attach to glass).

3) Gently pipette 3 ml of Hanks up and down 3 to 4 times only, dispensing the buffer above the pellet. This resuspends the pellet slowly. See Note 2, pg. 30.

4) Place the cell suspension in a 5% CO2 incubator at 37 ^{o}C until step C.b.5, leaving the caps slightly loose.

2. Washing

a. Centrifuge the cell suspension for 10 min. at 160xg at room temperature. Perform this step during the 15 min. incubation step in C.b.5.

b. Drain the Hanks supernatant as per step a.4.

c. Immediately resuspend the pellets in Opsono Buffer (Reagent P) using 4 ml per plate. Pellets should be resuspended with the same care as in step a.5.

d.. The final cell suspension should be homogeneous (single cell suspension with no visible clumps). These cells are ready to use in the assay. See Note 3, pg 30.

B. Serum Samples

1. Sample requirements

a. A minimum of 40 µl is needed to test a serum sample for opsonic activity against a single serotype of pneumococci. Sera should have no inhibitory agents, such as antibiotics, sodium azide, detergents, etc. The endogenous complement is inactivated at 56 ^{o}C for 30 min.

b. Human serum samples are ideally stored frozen at -70 ^{o}C, after collection, in small aliquots (~200 µl).

2. In-plate dilutions

a. Serum samples are tested at a starting dilution of 1:8 (in plate final dilution) and diluted in a two-fold dilution scheme. Appendix D. pg. 26.

b. Aliquot 20 µl of serum into each of the first wells (A1, A2, A3.....A12).

c. Samples are run in duplicate, so A1 and A2 are the same serum, A3 and A4 from a second serum sample, and so on until wells A9 through A12 are reached. These last four wells are filled with QC sera. A serum with known titer (low, medium or

high) is aliquoted into A9 and A10. Prediluted Sandoglobulin (to yield a starting dilution of 1:16 or 1:32, depending on the serotype) is aliquoted into A11 and A12.

d. Fill the other wells (B1-12 to H1-12) with 10 µl of Opsono buffer (Reagent P). Use a sterile reagent reservoir to hold the Opsono buffer. See Note 4, pg. 30-31.

e. Make two-fold serial dilutions of the samples using a multichannel pipeter adjusted to 10µl. Aliquot 10 µl from all the first row of wells avoiding air bubbles.

f. Place the pipeter into the second row of wells (all the way down and in contact with the 10 µl of Opsono buffer) and slowly pipette repeatedly, up and down 5 times, to mix the well contents.

g. Aliquot 10 µl from the second row and repeat step b.6 in the third row. Continuethrough row G.

h. Remove and discard the last four tips of the pipeter that were used to aliquot the QCsera. In this way, the last four wells of the plate (H9 through H12) will not receive any serum. These four wells are the complement control wells and should only contain 10 µl of Opsono buffer at this point.

i. Continue the dilution scheme through row H. Discard the remaining 10 µl in all 10 tips into the waste.

j. All wells should contain 10 µl. Row A will become a 1:8 dilution once the remainder of the reagents are added.

row B = 1:16

row C = 1:32

row D = 1:64

row E = 1:128

row F = 1:256

row G = 1:512

row H = 1:1024

G11 and G12 are 1:1024 dilutions for the Sandoglobulin QC, whereas H9 through H12 are the Complement control wells. Appendix D, **pg** 26.

k.. The final volume per well at the end of the entire assay will be 80 µl.

C. Preopsonization

1. Dilution of frozen bacterial stocks

a. Allow a frozen stock (See section VII, **pg**19-20) of the desired strain from the -70 ^{o}Cfreezer to thaw at room temperature for ~10 min.

b. Mix the vial by a quick vortex (2sec), take 100 µl and add to 0.9 ml Opsono buffer (this is a 10 -1 dilution).

c. Mix again and aliquot 100 µl from the 10-1 tube and add to 0.9 ml Opsono buffer (this becomes a 10-2 dilution).

d. Repeat the procedure to generate a 10-3 dilution. Most lot numbers of bacterial stocks will not need another 1:10 dilution. A smaller dilution may be required to adjust the number of bacteria to ~60 cfu in the T_0 control (1,000 cfu/well). See Note 5, **pg** 31. Double or triple the volume in the dilution scheme to accommodate the number of plates being run. Each plate requires 2 ml of diluted bacteria. Schemes vary according to each frozen strain and lot #. Use the attached table for guidance. See Appendix L, **pg** 45 and note 6, **pg** 31.

e. Prepare and plate T0 control:

1) Add 20 µl of the working dilution to 140 µl of opsono buffer.

2) Mix by vortexing and aliquot 10 µl at the top of a THYE plate. See Appendix C, **pg** 23. Repeat three times. Tilt the plate at a 45^{o} angle towards you. The liquid will run slowly down the plate. Label this plate T_0 and include the serotype being run.

3) Allow the liquid to completely adsorb into the agar before inverting the plate.

4) Incubate overnight at 37 ^{o}C in 5% CO2. ~60 cfu/aliquot are expected after overnight incubation.

2. Addition to plates

a. Use a multichannel pipeter set to 20 µl to add the diluted bacterial suspension from step C.a.4 to each of the 96 wells in a microtiter plate. Use a sterile reagent reservoir to hold the diluted bacterial suspension.

b. Replace the lids and incubate the microtiters plates at 37 ^{o}C in 5% CO2 for 15 min. This step allows the pre-opsonization of bacteria with diluted sera.

D. Complement Source

Baby rabbit serum is used as the complement source. Limited studies with human serum as the complement source, demonstrated that the opsonophagocytic titers of reference sera were not significantly altered (+ 1 dilution) by the use of either source of complement.

1. Frozen stocks of complement are stored at -70 ^{o}C. Thaw the vials of baby rabbit serum immediately before use. Ideally, the complement should be at ~4 ^{o}C before addition to plates. See Note 7, **pg** 31.

2. Following the incubation period (step C.b.2), add 10 µl of complement source (Baby Rabbit Serum) to all 96 wells using a multichannel pipeter.

3. When adding the complement, a 1 ml vial is enough for one microtiter plate. Thaw and combine all the vials necessary for the number of plates being run.

4. Pool the vials in a sterile reagent reservoir and quickly add the complement to all wells.

E. Phagocytosis with HL-60 Cells

a. Addition to plates

1. Add 40 ml of the resuspended cells from step A.b.4. into each well of a microtiter plate for the opsonophagocytic assay.

2. The total number of cells added to each well should be 4x105 in 40 ml. Each plate requires 4 ml. Use a sterile reagent reservoir to hold the cell suspension.

b. Incubation period

1. Replace the lids and tape them down to the plate.

2. Incubate the microtiter plates while rotating on a horizontal rotator shaker at 220 rpm at 37 ^{o}C, for a period of 45 min in room air.

F. Viable Counts

1. Using a multichannel pipeter, carefully mix (three times) the contents of the wells.

2. Plate a 5 µl aliquot from each well onto THYE plates (Medium 4) that have been pre-warmed to room temperature for 4 hours.

3. Plate column 1(A1 to H1) at the left side of the first THYE plate, by releasing the liquid above the

surface of the agar plate. See Note 8, **pg** 32.

4. Continue the plating procedure, column by column. Six columns can be plated on plate I and the last six columns (7-12) are plated on plate II. See Note 9, **pg** 32.

5. Allow the 5 µl spots to air dry (~5 min).

6. Invert the plates and incubate overnight at 37 ^{o}C in 5% CO2.

7. An aliquot of 5 µl corresponds to 1:16 of 80 µl (1,000 cfu/well), therefore the maximum expected colony count per aliquot is ~60 cfu. See Note 10, **pg**32.

8. Use a dissecting scope (15 x mag.) to count the colonies on plate with transmitted light. See Note 11, pg 32.

9. A viable count of the initial number of bacteria added at time zero (T_0) to each well is included per run for a particular serotype. Complement control counts and T_0 counts are expected to be close to each other (< 20%).

G. Opsonophagocytic Titers

The phagocytic titer is the reciprocal of the serum dilution with at least 50% killing, when compared to the average growth in the complement control wells (H9 through H12).

Occasionally, sera with high titers need to be retested at higher initial dilutions than 1:8 to determine the phagocytic titer. Serum samples with phagocytic titers <8 are reported as a titer of 4 for purposes of data analysis. See Note 12, pg 32-33.

H. Quality Control

a. Daily Controls

1. Time zero (T_0) control

Aliquot 20 µl of the working dilution of bacteria and add to a 140 µl of Opsono buffer, mix for 2 sec using a vortex (medium speed), plate 10 µl aliquots three times and tilt the plate. To do this, plate three individual 10 µl drops at the top of a THYE plate (label this plate T_0 and include the serotype being run) and tilt the plate at a 45^{o} angle towards you. The drops will run slowly down the plate, allow to dry (2 to 3 min) and incubate as above. ~ 60 cfu/aliquot are expected after overnight incubation at 37 ^{o}C in 5% CO2.

2. Complement (C') control

Four wells (H9 through H12) in each microtiter plate are used for this control. Add bacterial suspension (20 µl) to 10 µl of Opsono buffer. Add complement (10 µl) and HL-60 cells (40 µl). The average growth of the four complement control wells should be close to the T0 control (a variation of <20% is

acceptable). Occasional growth in the C' control wells (doubling) is preferable to killing. This control is prepared within the plate and at the same time that the samples are run. If the assay consists of several microtiter plates, use the average of the C' control wells in each plate to calculate the opsonophagocytic titers.

b. Additional Controls

The following controls may be included in a separate microtiter plate, when appropriate.

1. Complement source control

This control is run when a new lot of complement source is used to determine the possible presence of antibodies against pneumococci. It should be performed with every new serotype tested. Heat inactivate the complement source (100 µl) for 30 min at 56 ^{o}C. Add bacterial suspension (20 µl) to 10 µl of heat inactivated complement (new lot # to be tested).

Add active complement from the old lot # (10 µl) and HL-60 cells (40 µl). The complement control should be close to the T_0 control (a variation of <20% is acceptable).

2. Direct complement killing

This control is used to test for complement mediated killing in the absence of phagocytes.

With *S. pneumoniae* this control is not necessary if the strain is highly capsulated. Only rough strains will give direct complement killing. To run this control; add 20 µl of bacterial suspension to a 50 µl opsono buffer blank. Then, add 10 µl of complement source. Do not add phagocytes. This complement control should be close to the T_0 control (a variation of <20% is acceptable).

3. HL-60 growth control

This control is performed when a new serotype is used. Add 20 µl of bacterial suspension to a 20 µl Opsono buffer blank. Then, add HL-60 cells (40

µl), but no complement source is added. This control should be close to the control (a variation of <20% is acceptable).

4. Antibiotic control

This control is performed when a serum sample is suspected of having antibiotics. When various serotypes are being run with the same sample, perform the antibiotic control against one single serotype. Add 10 µl of test serum, 20 µl of bacterial suspension and 50 µl of Opsono buffer. Additional serum sample is required. In order to avoid disrupting the template format, these controls can be performed in a separate microtiter plate that is incubated along with the assay plates. This control should have growth or be close to the

T_0 control (a variation of <20% is acceptable). If no growth is obtained, the serum sample may have antibiotics and is not suitable for the opsonophagtocytic assay. This control isonly necessary when testing samples that are not part of a controlled study.

5. Complement independent killing

This control is performed to determine the level of killing that is independent of complement in a given sample. Add bacterial suspension (20 µl) to 10 µl of serum sample. Add heat inactivated complement (10 µl) and HL-60 cells (40 µl). Additional serum sample is required. The amount of killing obtained is sample dependent. Since the opsonophagocytic assay is standardized for complement mediated phagocytosis, this control is very seldom included.

H. Quality Control Sera

1. Sandoglobulin (human IgG) Sandoglobulin at 6% (Reagent T) is a commercial immune globulin pool composed of IgG (96% monomeric) antibodies in the native form. It is generated by cold alcohol fractionation from > 15,000 U.S. healthy donors. Sandoglobulin has a pH of 6.6 +/- 0.2 and is sterile. Sandoglobulin is aliquoted into small volumes (200-500 µl) and stored frozen at -70 oC. Prediluted Sandoglobulin is usually added to wells A11 and A12 of each microtiter plate, to yield a starting dilution of 1:16 or 1:32 (in Opsono buffer), depending on the serotype tested.

2. Known opsonic titers and IgG values for Sandoglobulin

Type	4	6B	9V	14	18C	19F	23F
Opsonic Titer	512	2048	256	2048	512	512	1024
IgG (mg/ml)	23.6	65.8	38.5	72.3	34.9	97.0	32.8

October 6, 2000

See Appendices J and K, pg 37-44.

3. QC sera with known titers

Quality control sera with known low, medium and high titers should be included in each assay. Include one QC serum per plate (wells A9 and A10). Any high volume serum (with a known reproducible titer) can be used as a QC serum.

V. UNDIFFERENTIATED HL-60 CELLS

Undifferentiated HL-60 cells (Reagent A) are purchased from the American Type Culture Collection (ATCC) at passage 20. HL-60 cells are shipped frozen (1 ml containing 5.6x106cells). The frozen stock can be expanded to a 100 ml volume (Medium 2) with ~6x105cells/ml. This generates enough cells to start splitting (the division of a high cell density culture into more diluted cultures) and inducing (the addition of N,N-dimethylformamide to enhance granulocytic differentiation) HL-60 cells for the

opsonophagocytic assay. It is recommended to keep three 100 ml cultures of undifferentiated cells and to feed or split them daily to maintain cell concentrations below 7x105 cells/ml. Two cultures are used for

differentiations and one for splitting into three new undiffentiated cultures at a higher passage number. **See Appendix I, pg 36.**

A. Expansion of Frozen Stock.

1. Thaw a 1ml frozen stock of HL-60 cells by placing the vial in a 37^{o}C water bath. See Note 13, pg 33.

2. Immediately dilute the thawed stock in 20 ml of Medium 2 (1:20) equilibrated at room temp.

3. Expand the cells in T-75 tissue culture flasks (Material 1) to a cell density of ~6x105cells/ml in Medium 2. See Note 14, **pg** 33.

B. Splitting of Undifferentiated Cells.

1. Divide the cells once a week, as follows:

a. Perform a viable cell count with 0.4% Trypan Blue (Appendix E) and calculate the volume required to start a new 100 ml flask of 3 at a cell density of 2x105 cells/ml. See Note 15, **pg** 33.

b. Harvest cells by centrifugation (160xg for 10 min at room temp) in 50 ml tubes (Material 3).

c. Resuspend cells by pipetting in 10 ml of the supernatant (10 % old culture medium

containing growth factors for the new passage).

d. Add c to 90 ml of Medium 2 (total volume; 100 ml).

2. Incubate at 37oC in 5% CO2 in an upright position with loose caps. Some lot numbers

grow better in the slanted position.

3. Cell densities are kept less than 7x105 cells/ ml. See Note 16, **pg** 33.

4. All culture flasks are incubated at 37^{o}C in 5% CO2 atmosphere in the upright position with loose caps (one and a half times from the closed position).

5. Feed the split cells daily.

6. To feed the cells, decant the medium without disturbing the cells that are settled at the bottom of the flask. Leave ~50% of the old medium in the flask and add fresh Medium 2 equilibrated at room temperature.

7. Cells can be split until very high passages are reached (up to passage #250 cells have been tested successfully). After this passage, start a new frozen stock at a lower passage (i.e.; 20 or 21).

VI. DIFFERENTIATION OF HL-60 CELLS

Undifferentiated cells grown to a cell density of 5-7 x105 cells/ml are used for differentiation. Differentiation is carried out in cultures with a 200 ml volume (T-150 flasks, Material 2) of Medium 2 by the addition of 100 mM N,N-dimethylformamide (Reagent E) as the chemical inducer.

1. Perform a viable cell count of the undiffer- entiated cell culture using 0.4% Trypan Blue. **Appendix E, pg 27.**

2. Calculate the volume of undifferentiated cell culture required to induce 200 ml of differentiated cells at 2x105 cells/ml. See Note 17, **pg** 34.

3. Centrifuge (160xg for 10 min at room temp) the calculated volume from step 2 in 50 ml tubes (Material # 3), ensure the tubes are balanced.

4. Discard most of the supernatant but allow for 20% of the old medium with growth factors to remain in the tube to resuspend into a single- cell suspension. See Note 18, **pg** 34.

5. Prepare tissue culture flasks with induction medium (160 ml per flask) while the cells are centrifuging in step 3.

a. Add 160 ml of Medium 2 into a 150 cm2 tissue culture flask using a 50 ml polypropylene plastic tube.

b. Add 1552 µl of DMF to the center of the medium. Use tips that are resistant to organic

solvents (i.e.; if using Rainin blue tips, add 776 µl twice).

c. Mix well to make a homogeneous suspension of the inducer.

6. Aseptically add the 40 ml of cell suspension from step 4 to the above medium (5.b), while

shaking with your hand the tissue culture flask.

7. Incubate for a period of 5 days at 37^0 C, 5% CO2 in the slanted position (taking care that the medium does not reach the cap area to avoid contamination) with the cap loose. There is no need to feed the cells during this period. See Note 19, **pg** 34.

8. HL-60 cells are 90-95% differentiated into granulocytes by 5 days, and are ready to be used as phagocytes with a typical yield of 6-8x105 cells/ml.

9. Differentiated cells are used at a 400:1 ratio (effector : target ratio) in the opsonophagocytic assays.

HL-60 Cell Count (cells/ml)	# of ml req./plate	# of Plates/200 ml culture of differentiated cells
3×10^5	130.6	1.5

4 x 10^5	98	2
5 x 10^5	78.4	2.5
6 x 10^5	65.3	3
7 x 10^5	56	3.5

See Note 20. **pg** 34-35 and Appendix I, **pg** 36.**VII. PREPARATION OF *S. PNEUMONIAE* STOCKS**

A. Growth of Bacterial Strains

1. All strains used should be highly encapsulated. To test for the presence of polysaccharide capsule, perform a Quellung test (9).

2. Use an overnight culture of pneumococcus grown in a Blood agar plate (Medium 6).

b. Adjust the suspension to a McFarland # 1 standard (~3 x 108 cfu/ml) in 0.5 ml of 0.85% saline solution. Use a sterile swab to harvest the bacteria growing on the surface of the plate.

c. On a glass slide place: 10 µl of Omni serum (Reagent J), 10 µl of 0.3% Methylene Blue and 10 µl of the adjusted bacterial suspension.

d. Mix carefully with the pipette tip and place a coverslip on top.

e. Read under oil immersion with the condenser slightly lowered from its highest position and the diaphragm almost closed to improve refractivity of the capsule. The bacterial cells will stain blue and the capsules will appear as a clear halo surrounding the diplococci. The bacterial strain is considered highly encapsulated if the capsules are of the same diameter as the cell or larger.

f. The amount of agglutination (positive reaction with the serum) is read under the 10x objective and recorded as -, +, ++, +++ or ++++. Highly encapsulated strains would give a +++ or ++++ reaction.

2. Prepare an overnight culture of *S. pneumoniae* grown on a Blood agar plate at 37 ^{o}C and 5% CO2.

3. Inoculate 5 ml of Todd-Hewitt broth and 0.5% yeast extract (Medium 4) with a few well isolated colonies from step 2.

4. Incubate overnight at 37 ^{o}C and 5% CO2.

5. Inoculate a 50 ml flask of Medium 4 with a 500 µl aliquot from step 3.

6. Incubate for 5 to 8 h, at 37 ^{o}C and 5% CO2 to an OD420 = 0.5 to 0.6 (mid-log growth phase for the majority of strains tested using a 13 mm [outer diameter] path length).

B. Freezing

1. Add 7.5 ml of sterile glycerol to each 50 ml culture at OD420 = 0.5 to 0.6.

2. Dispense in 1 ml aliquots into cryovials (Material 7).

3. Flash freeze in a 95% ethanol-dry ice bath.

4. Keep frozen vials at -70 ^{o}C for further use (up to 18 months).

5. Viable counts of randomly chosen aliquots are performed to determine the concentration of pneumococci, before and after the freezing protocol. For the viable counts:

a. Prepare 7 or 8 10 fold dilutions of the stock in sterile 0.01 M PBS, pH 7.2

b. Plate a 100 µl from the 10 -6, 10-7 and 10-8 dilution on the surface of Blood agar plates (Medium 6).

c. Incubate overnight at 37 ^{o}C and 5% CO2.

d. Count the number of alpha hemolytic colonies.

6. Determine dilution schemes for all strains to assure the use of ~1000 cfu/20 µl in the functional assay:

a. Dilute the frozen stock up to the 10-3 and 10-4 dilutions.

b. Prepare a T0 tube from each of these two dilutions as follows: 140 µl of Reagent M + 20 µl of the test dilution (10-3 or 10-4).

c. Plate 10 µl aliquots three times. Tilt the plate to spread the aliquot.

d. Allow the aliquot to adsorb into the agar for 5 min and incubate as above.

e. Count the colonies in each streak of growth and find the average count for each dilution. The ideal number of colonies is 60 + 5 cfu. However, the most likely scenario is a higher count in 10-3 and a lower count in 10-4.

f. Calculate the dilution factor needed to bring the count down to 60 cfu. This becomes the dilution scheme for that strain's frozen lot. See Note 21, **pg** 35 and Appendix L, **pg** 45.

APPENDICES

A. MATERIALS

1. Tissue culture flasks (T-75), Corning, Cat. # 25115-75. Can substitute vented flasks, if preferred.

2. Tissue culture flasks (T-150), Corning, Cat. #: 25120-150.

3. Centrifuge tubes, 50 ml, polypropylene, Corning Cat. # 25330-50.

4. Rainin tips RT-20 (0-200 µl, yellow), sterile.

5. Rainin tips: 1 ml, blue; sterile. Must be resistant to organic solvents.

6. Plastic pipetes, 5, 10 and 25 ml sterile and individually wrapped.

7. Cryovials, 2 ml, polypropylene, Nalgene, Cat. # 5000-0020.

8. Dry ice.

9. Freezer box (a styrofoam mailing box with lid works find).

10. Freezer storage boxes (Nalgene).

11. Microtiter plates, round bottom, sterile with lids. Costar, Cat. # 3799.

12. Reagent reservoirs (20 ml), sterile, Costar , Cat. # 4870.

13. Sterile plates, 100x15 mm, square grid, Falcon, Cat. # 1012.

B. EQUIPMENT

1. Incubator at 37 ^{o}C under a 5% CO2 atmosphere.

2. Incubator at 37 ^{o}C, room air.

3. Inverted microscope with 10x objective or higher.

4. Light microscope with 10x and 100x objectives.

5. Pipeteman (1000, 200 and 20 µl).

6. Multichannel pipette (8 to 12 channels and a 5 to 50 µl range).

7. Disecting scope.

8. Table top centrifuge (up to 500 x g range) at room temperature.

9. Horizontal rotator shaker (up to 250 rpm range) to fit inside Equipment 2.

10. Water bath at 37 ^{o}C.

11. Autoclave.

12. Hemacytometer (Newbuer counting chamber).

C. MEDIA AND REAGENTS

MEDIA

1. Medium 1, RPMI 1640 medium containing 1% L-glutamine but without Phenol red

(Life Technologies, Cat. # 11835-030).

2. Medium 1 with heat inactivated 20% fetal bovine serum (FBS, Reagent D).

3. Medium 2 with antibiotics; add 5 ml of Reagent C per 500 ml of Medium 2.

4. Todd-Hewitt Broth with 0.5% yeast extract.

5. THYE agar plates; prepared with Medium 4 and 1% Purified Agar, Difco, Michigan, Cat. # 056001

6. Blood agar plates (5% sheep's blood) purchased from BBL (Becton Dickinson

Microbiol. Systems), Cat. # 21239/21261.

PREPARATION OF THYE AGAR PLATES

1. Prepare 250 ml vials of Todd-Hewitt broth with 0.5% yeast extract (Medium 4). For 1L of medium: add 30 g of Todd-Hewitt broth (Difco) and 5 g of Yeast Extract (Difco) to 1000 ml of ETF water (type 1); autoclave 15 min at 121^{o} C and 15 psi.

2. Add 2.5 g purified agar to each 250 ml vial.

3. Loosen caps (allowing evaporation)and place in the autoclave for 15 min at 121^{o} C and 15 psi.

4. Remove from autoclave and place in a 56^{o} C water bath for 30 min.

5. Replace the covers and pour approx. 20 ml into each square petri dish (Material 13).

6. Do not disturb the plates until the medium has solidified. Close covers.

7. Allow the plates to sit inside hood overnight to check for sterility.

8. To prevent plates from drying out, store them in an inverted position inside a plastic bag at 4^{o} C up to 3 months.

REAGENTS

A. Tissue culture cell line: HL-60 promyelocytic leukemia (CCL240, ATCC, Rockville, MD).

B. Highly encapsulated strains of *S.pneumoniae* in frozen stocks with known concentration and dilution schemes.

C. Antibiotics (10x penicilllin-streptomicyn solution), Life Technologies, Cat. #15140 - 023.

D. Fetal bovine serum (Hyclone).

E. N,N-Dimethylformamide 99.8% purity, Fischer, Cat. #: D131-1.

***Caution**: Organic solvent should be handled under good ventilation (biological safety cabinet or chemical hood), with gloves, lab. coat and goggles. Explosive and possible carcinogen. Avoid direct inhalation of fumes.**

F. Giemsa stain:

Giemsa powder, 0.5 g, Glycerol, 33 ml, absolute methanol (acetone-free), 33 ml. Dissolve the Giemsa powder in the glycerol at 60^{o} C for 2 hr. Add the methanol, mix thoroughly, and allow to stand. Store sediment-free reagent at room temp.

G. Nitrobluetetrazolium (NBT) solution:

NBT at 1mg/ml in 10% FBS and 90% PBS (0.01M, pH 7.2). Keep in the dark at 4^{o} C. It should be an amber color and clear.

H. 0.3% solution of Methylene Blue

I. 0.85% saline solution

J. OMNI serum (rabbit anti-pneumococcal serum), Statens Seruminstitut, Copenhagen, Denmark.

K. Differentiated HL-60 cells

L. Control serum (Sandoglobulin 6%), Sandoz Pharmaceuticals, Cat. # NDC 0078-0124-60 reconstituted in diluent NDC 0078-0125-37 to the 6% mark. East Hanover, NJ.

M. Hank's balance salt solution, Life Technologies, Cat. # 14025

N. Hank's balance salt solution without Ca++ and Mg++, Life Technologies, Cat. # 14175

O. 1% Gelatin solution (sterile).

P. Opsono buffer: Reagent M with 10% of Reagent O.

Q. 0.4% Trypan Blue solution in 0.01 M PBS, pH7.2.

R. Ethanol, 95%.

S. Baby rabbit (3-4 week old) serum, sterile. Pel-Freez Biologicals, Cat. # 31038-2 for 100 ml volumes.

Upon arrival, thaw 100 ml of baby rabbit serum, keep the flask in ice after thawing and dispense 1 ml aliquots in cryovials for flash freezing in a 95% ethanol- dry ice bath. Frozen aliquots are kept at -70 ^{o}C for further use.

T. Endotoxin-free water, type 1 water, sterile.

U. 0.01 m PBS, pH7.2. Life Technologies, Cat. # 93-0222DK.

V. Glycerol, Sigma Chem. Co. Cat. # G-9012. Sterilize by autoclaving for 15 min at 121^{o} C and 15 psi.

E. VIABLE CELL COUNTS

1. Use a hemacytometer.

2. Dilute cells 1:5 , as follows:

10 ml of Hanks (Reagent N)

30 ml of 0.4% Trypan Blue solution (Reagent Q)

10 ml of HL-60 cells (undifferentiated or differentiated)

3. Mix and load 10 ml into the top chamber.

4. Count the four outer squares and the center square in the grid pattern.

5. Multiply the number obtained from the count by 104.

6. Example: Cell count of one chamber

(5 squares) = 56

56 x 104 = 5.6 x 105 cells/ml.

7. Viable cells are greenish in apparency and refractile, whereas dead cells are dark blue.

Differentiated cells may not look perfectly round.

F. LIST OF SUPPLIERS

ATCC, 12301 Parklawn Drive, Rockville, MD 20852 U.S.A.

ph: (800) 638 6597

BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD 21030 U.S.A.

ph: (800) 638 8663, Fax: (410) 584 7785

Fischer Scientific, Fairlam, NJ 07410 U.S.A.

ph: (201) 796 7100, Fax: (800) 926 1166

Life Technologies. P.O. Box 68, Grand Island, NY 14072-0068 U.S.A.

ph: (800) 828 6686, Fax:(800) 331 2286

Pel-Freez Biologicals. 9099 N. Deerbrook Tr. Brown Deer, WI 53223 U.S.A.

ph: (800) 558-4511, Fax: (414) 357-4518

Sandoz Pharmaceuticals Co. 59 Route 10, East Hanover, NJ 07936-1080 U.S.A.

ph: (201) 503 7500, Fax: (201) 503 8265, alternatively

Bayer Co. Pittsburgh, PA, ph: 1 800 288 8370

Sigma Chemical Co. P.O. Box 14508, St. Louis, MO 63178 U.S.A.

ph: (314) 771 5750, Fax: (800) 325 5052

Statens Seruminstitut. 5, Artilleriveg. DK-2300 Copenhagen S., Denmark.

ph: 45 32 68 32 68, Fax: 45 32 68 38 68

G. OPSONINS AND RECEPTORS

The ability of protective antibodies to bind the target bacterium depends upon recognition of the most outer molecules on the surface of the bacterium, i.e., capsular polysaccharides and surface proteins. The Fc regions of adjacent antibodies allow for the fixation of complement. The deposition of C3b or iC3b act as opsonins (14). The amount of

complement that is fixed by different IgG subclasses varies. IgG1 and IgG3 fix

complement more efficiently than IgG2 and IgG4. However, IgG2 may be able to fix

complement at high epitope densities and is also the best activator of the alternate pathway at high antibody concentrations (6). Antibodies (mainly IgG2) against the cell wall

polysaccharide (CPS) can represent the majority of the signal measured by ELISA, and are

considered to be non-protective (8). In the case of opsonophagocytosis, the interference byCPS antibodies is minimal. Vioarsson et al. reported this lack of interference of CPS in the

opsonic activity against S. pneumoniaetype 23 (14). Other factors, such as the type of

receptors expressed on the surface of the phagocytic cells may also play a role in the quantitation of functional antibody, for example IgG2 binds poorly to the Fc gamma RIIa, HR allotype, although it binds with the same affinity as IgG1 to the LR allotype. IgG3 has the highest affinity of all IgG subclasses for both the LR and the HR allotypes. IgG1 and IgG3 have equally high affinity for the RIIIb allotypes, whereas IgG2 and IgG4 have no affinity for this Fc gamma receptor. Although, HL-60 cells are of the R/R phenotype which is of low affinity, in the presence of complement the opsonophagocytic activity measured have been shown to be as efficient as the activity obtained with PBLs from human donors.

H. NOTES

1. Example:

Cell count: 5.6 x 105 cells/ml

Volume/bottle: 200 ml of induced cells

# of cells required/well: 4 x 105 cells

# of wells per plate: 100 (4 extra wells for pipetting errors)

Use the formula: Cells needed/well x 100 = total # of ml per plate.

Cell Count:

4 x 105 cells/ well x 100 wells = 70 ml.

5.6 x 105 cells/ml

Therefore, 70 ml of induced cells at a cell density of 5.6 x 105 cells/ml are needed for one assay plate. In this example, 200 ml of this particular bottle will only be enough to run 2.9 plates. The cell density of each 200 ml bottle of induced cells needs to be determined individually. Cells densities vary considerably between bottles of the same lot (date) of induction.

2. The cells start to come into suspension fairly quickly, avoid pipetting any clumps up and down. Pipetting should be gentle and slow. Take care and time to do this in order to avoid lysing of cells by harsh pipetting. Combine tubes for the final centrifugation. The cells from 4 plates end up in two tubes with 20 or 30 ml per tube. If medium with antibiotics is used during induction, the cells need to be washed one more time to remove the antibiotics, prior to the addition of Opsono buffer.

3. Washing steps 1 through 4 should not be performed until the preopsonization period so that cells do not remain in buffer with Ca++ and Mg++ for >10 min (increases the chance of clumping). Fifteen min is all the time required to centrifuge the cells and resuspend them. They will be ready to add to the microtiter plates after the addition of the complement source.

4. This buffer is prepared fresh daily (a 50 ml volume is sufficient to run 4 microtiter plates from start to finish). For a 50 ml volume, add 5 ml of 1% gelatin (Reagent O) + 45 ml of Hanks with Ca++ and Mg++ (Reagent N). Keep at room temperature. While running the assay, it is helpful to fill the first plate with Opsono buffer, and then add the serum samples to the first row of wells. Serially dilute the first plate before moving onto the second plate. Do not place the plates on the screen of the cabinet where the air flow is stronger; keep the lids on top of the plates (closed). Be ready and prepared to work

quickly and without interruptions. Following these recommendations, the volume per well

should be close to 10 µl throughout the dilution process. Work with one plate at the time for serial dilutions.

5. Example:

strain 9V (DS400-92) is diluted as follows:

10-1, 10-2, 10-3 +3.6 ml of Opsono buffer. To the 10-3 dilution, 3.6 ml of Opsono buffer are added to generate a final volume of 4.6 ml in the last tube. This becomes the working dilution for strain 9V. 4.6 ml gives enough volume to add 20 µl /well (1,000 cfu) to 2 microtiter plates.

6. Prepare these dilutions immediately before use. Do not let them sit for more than 10 min at room temperature. Otherwise, the bacteria will start growing and the dilution will not give ~60 cfu in the T0 control. If you need to prepare more than three different strains at once, it is recommended to place them on ice until ready to use. Add disinfectant to the reservoir containing the bacterial suspension before discarding it into the waste.

Note 7. Upon arrival, thaw the 100 ml of Baby Rabbit Serum by placing in a 37^{o}Cwater bath until ~90% of the serum is in the liquid form. Keep the flask on ice after partially

thawing the serum and dispense 1 ml aliquots in cryovials for flash freezing in a 200 proof

ethanol-dry ice bath. Frozen aliquots are kept at -70oCfor further use. Each vial should

only be thawed out once. Do not refreeze. Discard any unused amount after thawing out a

vial. Complement is not stable at room temperature and has only a very short half life at

4oC

8. Try not to punch holes in the agar with the pipet tips and make sure that all tips delivered 5 µl onto the agar. Eight small drops, equally spaced should be visible on the surface of the agar. If any of the drops join together, keep track of the well number and repeat on an extra plate that can be used for repeats.

9. If you are running several microtiter plates, a labeling system is necessary to identify each of the two THYE plates corresponding to each microtiter plate. Plate in the same order that you set up the microtiter plates.

10. Viable colony counts are performed early in the morning to avoid overgrowth of colonies on the plate.

11. Bacterial colonies appear whitish to translucent on the surface of the agar. The edges are smooth and the centers of the colonies may be sunk in (donut-like). Some strains may present various colony types, without being contaminated (colony variability; from opaque to shiny and small to large). Some human sera may clot slightly in the wells that have the highest concentrations of serum. Special care has to be taken when plating these samples. Mix well before plating the aliquots, tips may get clogged and may need to be replaced (this has been observed in two sera out of ~100 samples). Cell debris from the HL-60 cells, could be confused as bacterial colonies by an inexperienced technician. However this debris is irregular in shape and tends to run together like a maze. If holes are punched into the agar at the time of plating, the bacterial colonies will grow within the agar with highly irregular edges, making the count extremely inaccurate.

12. This example was randomly picked among hundreds of assays performed.

1:8 1:16 1:32 1:64 1:128 1:256 1:512 1:1024 C' T0

6 16 7 13 20 21 35 44 72 62 cfu

92% 78% 90% 82% 72% 71% 51% 39% ---> ki lling vs C' control.

The titer was reported as 512 for serum 95017002.

13. Do not submerge the edge of the vial, it could lead to contamination. The quicker the thawing procedure, the higher the recovery of viable cells.

Note 14. 20 ml of Medium 2 are added to the 1:20 dilution from step 1 every Monday and Friday; by the end of two weeks the concentration should be ~6x105cells/ml in a 100 ml final volume.

Note 15. Antibiotics can be added to the differentiation medium, however a total of two full washes have to be performed in Hanks without divalent ions to avoid carry over into the functional assay with pneumococci. Final resuspension is in Opsono buffer, which contains the divalent ions.

Note 16. Every time that the cells are divided at 2x105 cells/ml, a new passage number should be recorded on the culture flask. Usually on Mondays and Fridays the cells are split this low. On other week days they can be feed or split high (to ~3.5x105cells/ml) the day before induction (Tuesdays, Wednesdays, and Thursdays)

Note 17. Example:

Uninduced cell count = 5.9x105 cells/ml,

# of ml required for 200 ml differentiated cells at 2x105 cells/ml = 67.8 ml.

This number is obtained by :

2x105 cells/ml x 200 ml = 67.8 ml

5.9x105 cells/ml

18. If you wait too long to add the cells into the final bottle, the medium will start to turn alkaline, since we are no longer using phenol red as an indicator, pH changes are not visible but are still harmful to the cells. Avoid keeping the cultures outside of the CO2incubator for prolonged periods of time.

19. Pellets of undifferentiated cells vary according to the initial cell density of the undifferentiated culture, as judged by viable counts with 0.4% Trypan Blue exclusion. By

adding 1552 µl of DMF in a 200 ml final volume, the final concentration of DMF is 100 mM.

20. Comments about Differentiation

Cells are used with highly reproducible results at 5 days of induction. No additional changes of medium are necessary. Differentiated cells will start to elongate and take a sickle-cell type appearance that can be observed under the inverted microscope with a 10x objective. Final differentiation can be determined by Giemsa staining of a thin cell smear, counting the differentiated cells vs. the undifferentiated cells. Differentiated cells are neutrophil-like with a multilobar nucleus and a fairly clear cytoplasm, bands (metamyelocytes) and monocyte-like cells with a pronounced invagination of the nucleus (kidney bean shape) can also be considered phagocytic. Undifferentiated cells are mainly promyelocytes or myelocytes. Promyelocytes appear with a heavily stained nucleus that covers the majority of the cell. The cytoplasm is hardly visible and a nucleolus is apparent in most cells as a big hole or vacuole-like structure inside the nucleus. Myelocytes are larger in size and have a distinct cytoplasm. However, the nucleus has not started to invaginate yet. The nucleolus is not apparent anymore. These cells are not phagocytic and should be considered undifferentiated. The level of activity of the cells can also be tested by the measurement of superoxide dismutase with the nitroblue-tetrazolium test (NBT). Qualitative and quantitative NBT assays can be used (13).

21. A count of 60 cfu is equivalent to ~1000 cfu in 20 µl since 60 x 16 (dilution factor in the T0 control) = 960. The same frozen lot per strain should be used throughout the entire investigation.

Addendum Note . A flow cytometric protocol for opsonophagocytosis has been developed also at the Centers for Disease Control and Prevention. See, publication #3 in page 48.

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